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Multiplex Screening of Persistent Organic Pollutants in Fish Using Spectrally Encoded Microspheres

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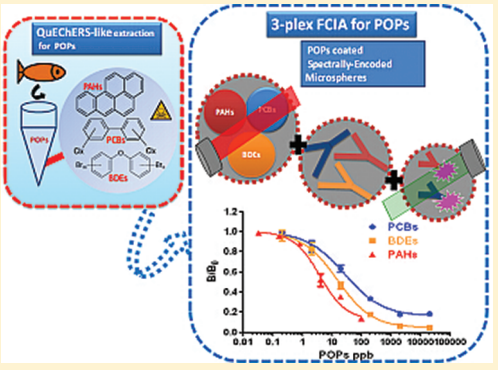
Supporting Information

ABSTRACT: Persistent organic pollutants (POPs) are environmental and food-related contaminants of global public health concern and known to be carcinogenic and endocrine disruptors. Their monitoring is essential, and an easy-to-use, rapid, and affordable multianalyte screening method with simplified sample preparation can be a valuable tool prior to instrumental analysis. For this purpose, a flow cytometric immunoassay (FCIA), based on a spectrally encoded microbeads technology, was developed for the multiplex detection of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (BDEs) in buffer and fish extracts. The sensitivities of the assays in the three-plex FCIA format were similar to the individual FCIAs for the marker compounds benzo[*a*]pyrene (BaP), 3,3′,4,4′-tetrachlorobiphenyl (PCB77), and 2,2′,4,4′-tetrabromodiphenyl ether (BDE47) in buffer with IC_{50} values of 0.4, 20, and 2 $\mu g\ L^{-1}$, respectively. Apart from the three markers, we could detect at least 14 other POPs. Extracts of fish with different fat content, prepared with a simplified extraction and cleanup procedure, had an insignificant influence on the overall three-plex FCIA performance, with the exception of some impact on the PAHs detection. The performance of the three-plex FCIA, in combination with the simple extraction procedure, is adequate for regulatory control in accordance with the required limits.



These days, it is known that the consumption of food contaminated with persistent organic pollutants (POPs) can cause acute intoxication incidents after high levels of exposure. Furthermore, diseases can appear after low-level chronic exposure of these chemicals.[1,2] During the past years, various contamination incidents with POPs in food have been reported.[2−6] Polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (BDEs), and polycyclic aromatic hydrocarbons (PAHs) represent the major groups of persistent toxicants not only in the environment but also in high fat content foods such as fish, due to their lipophilicity.[4,7−9] Consumption of food contaminated with POPs is the major exposure route for humans compared to other ways, such as inhalation and dermal contact.[10,11] To reduce health risks from the exposure to POPs, both the European Commission (EC)[12] and the United States Environmental Protection Agency (U.S. EPA)[13] established monitoring programs according to the Stockholm Convention[14] on POPs. The EC set maximum levels (MLs) for the sum of dioxins and dioxin-like PCBs and benzo[*a*]pyrene (BaP)[15] in various foods and animal feed. Apart from BaP, seven other carcinogenic PAHs[16] are under evaluation to be included as indicators of PAHs occurrence and toxicity in food. So far, no regulatory limits have been established for the BDEs; however, under the European regulatory framework,[17] the authorization procedure foresees that the utilization of BDEs can be subject to an authorization requirement.[18]

Instrumental analytical techniques that have routinely been used to detect POPs[7,19−22] are quite sensitive, specific, and irreplaceable in terms of identification power. However, they require costly equipment, skilled personnel, and they are time- and labor-intensive. In vitro bioanalytical assays, such as the aryl hydrocarbon hydroxylase (Ahh)/ethoxyresorufin-*O*-deethylase

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(EROD) bioassay and the chemical-activated luciferase gene expression (CALUX) bioassay[23−26] were developed to analyze Ah receptor agonists, such as several dioxin-like PCBs, as well as some BDEs and PAHs. These assays are cheaper compared to instrumental analytical techniques; however, recombinant cell culture lab facilities are necessary. Several enzyme-linked immunosorbent assays (ELISAs) were developed for the detection of PCBs, PAHs, and BDEs separately[27−34] but, so far, no attempts at simultaneous multiplex analysis have been made.

Rapid screening methods which are simple, inexpensive, fast, sensitive, have high throughput, and the possibility of detecting multiple POPs simultaneously are greatly needed. A new open platform in food analysis that enables the rapid analysis of a large number of samples for multiple analytes is the superparamagnetic (MagPlex) spectrally encoded microbead (xMAP) technology combined with flow cytometry (Luminex), which has been described for the analysis of several contaminants in food.[35,36]

In the present work, we utilized this technology for the development of a three-plex flow cytometric immunoassay (FCIA) for the detection of three major POPs using BaP, 3,3′,4,4′-tetrachlorobiphenyl (PCB77), and 2,2′,4,4′-tetrabromodiphenyl ether (BDE47) as marker compounds. After the characterization of the three-plex FCIA, the performance was tested in fish extracts prepared with a simplified and fast sample extraction and cleanup based on the quick, easy, cheap, effective, rugged, and safe (QuEChERS)[37] approach.

EXPERIMENTAL SECTION

Reagents, Materials, and Equipment. The mouse monoclonal antibody (Mab) against BaP (two batches of Mabs of different purities) and the BaP conjugated to bovine serum albumin (BaP−BSA) were purchased from the Technical University of Munich (Munich, Germany). Four rabbit polyclonal antibodies (Pabs) against BDE47 (PabBDE47 nos. 122, 123, 124, and 125) and the BDE47 conjugated to BSA (BDE47−BSA) were kindly offered by Dr. Weilin L. Shelver of the USDA (Fargo ND, U.S.A.). The two rabbit Pabs against PCB77 (PabPCB77-3TG and PabPCB77-5TG) and the PCB77 conjugated to ovalbumin (PCB77−OVA) were gifts from Dr. Milan Franek of the Veterinary Research Institute (Brno, Czech Republic). The goat antimouse and goat antirabbit R-phycoerythrin (PE) conjugates were from Prozyme (San Leandro, CA, U.S.A.).

Most of the stock standard solutions of PAHs, PCBs, and BDEs ($n = 51$) (Table S-1 in the Supporting Information) were supplied in the water-miscible organic solvents dimethyl sulfoxide (DMSO), methanol (MeOH), or acetonitrile (ACN) by AccuStandard (New Haven, CT, U.S.A.). Dr. Ehrenstorfer Laboratory (Ausburg, Germany) supplied the dibenzo[*a*,*e*]pyrene, cyclopenta[*c*,*d*]pyrene, 5-methylchrysene, benzo(*c*)fluorine), and BDE47. Aroclor mixtures 1232, 1242, 1248 were provided by Supelco (Bellefonte, PA, U.S.A.).

Protein LoBind tubes (1.5 mL) were supplied by Eppendorf (Hamburg, Germany), and the LoBind 96-well microplates were from Greiner Bio-One B.V. (Alphen a/d Rijn, The Netherlands). The *N*-hydroxysulfosuccinimide sodium salt (sulfo−NHS) was provided by Fluka Analytical (Steiham, Switzerland). *n*-Hexane-dichloromethane and silica (0.063−0.200 mm) were supplied by Merck (Darmstadt, Germany), and ethyl acetate was from Sigma-Aldrich (Steinheim, Germany). Magnesium sulfate and sodium chloride for the QuEChERS-like extraction were delivered from Sigma-Aldrich and Lach-Ner (Neratovice, Czech Republic), respectively. All other reagents not specified above were from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). The blank and the different POPs-contaminated fish extracts were caught at the locality Vranany on Vltava (Moldau) river located downstream from Prague industrial region and analyzed with gas chromatography/mass spectrometry (GC/MS) by the Institute of Chemical Technology Prague (Prague, Czech Republic). The equipment used in this study is described in the Supporting Information.

Procedures. *Purification of the PabPCB77.* PabPCB77 antiserum was affinity purified, in order to remove a BDE47 cross-reacting fraction, by incubating the antiserum (final dilution 1/1000) for 1 h with BDE47−BSA-coated superparamagnetic microbeads (final number of beads used ∼500 000). After the incubation, the BDE47 cross-reacting fraction was removed with the help of a magnetic separator. The affinity-purified PabPCB77 was always prepared fresh prior to the analysis.

Three-Plex FCIA. The protocol for the three-plex FCIA was similar to the single-plex FCIA protocol with the exception of the application of mixtures of reagents (antibodies, beads, and labels), instead of individual reagents. For the three-plex FCIA analysis, the POPs standard dilution series was prepared in the working buffer (5.4 mM sodium phosphate, 1.3 mM potassium phosphate, 150 mM sodium chloride pH 7.4 (PBS) with 2% DMSO and 0.7% BSA) of which 40 μL was combined with 40 μL of 50% DMSO in the well of a low-binding 96-well microplate. To obtain a similar DMSO concentration, the sample extract in DMSO was diluted with working buffer (1:1; v/v) and 40 μL of this diluted sample extract was combined with 40 μL of working buffer. After that, 20 μL of the mixture of the three different antibodies in PBS, the Mab against BaP (in a final dilution (fd) of 1/1000) and the two Pabs against PCB77 (affinity purified and with a fd of 1/1000) and BDE47 (fd 1/250), was added. The mixture was incubated for 15 min, and then 10 μL of the mixture of the three different POP-coated microbeads (the protocol for the preparation of the POPs-coated beads is described in the Supporting Information) was added to the well providing at least 1000 microbeads per set per test. The mixture was incubated for 45 min at room temperature in the dark on a plate shaker. After the incubation, the unbound bioreagents were removed by three washing steps with PBS, using the magnetic plate carrier of the automated wash station. Next, 25 μL of the mixture of the antimouse−PE and antirabbit−PE in PBS (both in a fd of 1/500) was added, followed by 100 μL of PBS, and then the mixture was incubated for 20 min in the dark, followed by one washing step with PBS. The microbeads were resuspended in 100 μL of PBS, and finally, the measurement in the Luminex was read for 20 s using 50 μL per well.

To prepare dose−response curves in buffer or in blank fish extract, a dilution series of the three POPs (0.01−1000 μg L^{-1}) was prepared either in the working buffer or in the diluted blank fish extracts. As negative controls, we used buffer or dilutions of blank fish extract (1:1; v/v). The contaminated fish extracts were diluted in the same way as the spiked blank samples.

POPs Extraction from the Fish Samples and GC/MS Analysis of the Fish Extracts. The extraction protocol for fish samples followed a previously described procedure.[38] Briefly, 10 g of homogenized fresh fish (5 g of homogenized smoked fish) muscle tissue was mixed in a polypropylene tube with 5 mL of distilled water and 10 mL of ethyl acetate and shaken vigorously for 1 min. Subsequently, 4 g of magnesium sulfate and 2 g of sodium chloride were added. After 1 min of shaking, the tube was

centrifuged for 5 min (11 000 rpm), and finally an aliquot of 5 mL in the case of fresh fish (for the PCBs and BDEs extraction) and 4 mL in the case of smoked fish (for the PAHs extraction) from the ethyl acetate layer was removed and evaporated under a gentle flow of nitrogen. If no extra cleanup was needed, the residue was dissolved in 1 mL of DMSO. For the noncontaminated fish spiked with different Aroclor solutions, 10 g of fish muscle tissue was spiked 15 min prior to the extraction with 50 μL of original standards (concentration 200 000 ng mL^{-1}) of Aroclor 1232, 1242, and 1248, respectively, resulting in 1000 μg of the different Aroclors per kg of fish tissue. For the cleanup, the residue was redissolved in 1 mL of hexane and this solution was introduced into a laboratory-made silica solid-phase extraction (SPE) column (Pasteur pipet filled with glass wool, 1 g of silica, and ca. 0.2 g of sodium sulfate) which was preconditioned with 6 mL of hexane/dichloromethane (3:1, v/v) followed by 4 mL of hexane. After the sample load, the analytes were eluted with 10 mL of hexane/dichloromethane (3:1, v/v). The eluate was evaporated by a rotary vacuum evaporator, and the residual solvents were removed by a gentle stream of nitrogen gas. The residue was dissolved in 1 mL of DMSO. The analysis of the fish extracts by GC/MS is described in the Supporting Information.

RESULTS AND DISCUSSION

Development of the Single-Plex FCIAs Prior to the Three-Plex FCIA. The research started with the development of the individual (single-plex) FCIAs for each of the three target model analytes (BaP, PCB77, and BDE47) using the different available immunoreagents (antibodies and protein conjugates of the three POPs). The competitive inhibition format of the single-plex FCIAs was based on previously described work with PAHs.[36] Briefly, we coated PCB77−OVA, BDE47−BSA, and BaP−BSA protein conjugates to three different sets of spectrally encoded microbeads. A Mab against BaP and Pabs against PCB77 and BDE47 were tested during the development of the single-plex FCIAs. For the measurement of the bound antibodies to the coated beads, goat antimouse or antirabbit Pabs labeled with PE were used. All these available immunoreagents were tested for their optimum dilutions in combination with different sequential incubation conditions. The water solubility of PCBs, PAHs, and BDEs is very low, so they are usually extracted from food matrixes using organic solvents.[39,40] In order to increase the solubility of POPs and to avoid adsorption to the well plate, the final concentration of DMSO in the well was around 20%, resulting in no influence on the sensitivities of the three single-plex FCIAs. Also in earlier described immunoassays for various POPs, several organic solvents such as DMSO, ACN, and MeOH were used up to 50% without a significant influence on the assay sensitivities.[29,30,32,34] The criteria used to evaluate the optimization process were the maximum median fluorescence intensities (MFIs), aiming for around 2000 MFIs for the blank measurements (the maximum responses) in a competitive inhibition format, the dynamic ranges, and the lowest IC_{50} values for each of our target analytes. The optimum combinations for each individual FCIA were PabPCB77-3TG (fd 1/4000) with PCB77−OVA-coated beads for the detection of PCBs, MabBaP (fd of 1/1000) with BaP−BSA-coated beads for the detection of PAHs, and PabBDE47122 (fd 1/1000) with BDE47−BSA-coated beads for the detection of BDEs. The normalized PCB77, BaP, and BDE47 dose−response curves obtained in the single-plex FCIAs are given in Figure 1, where the B_0 is the maximum MFI of the blank measurement and B is the MFI obtained with the different analyte concentrations. The IC_{50} values (the concentrations of the analytes at 50% inhibition of the maximum responses) for each analyte were 20 ± 2, 2 ± 0.2, and 0.3 ± 0.1 μg L^{-1} for PCB77, BDE47, and BaP, respectively. The IC_{50} values of the single-plex for PCB77 and BaP in buffer were in good agreement with those obtained with the ELISA using the same antibodies,[32,34] only the sensitivity for BDE47 was higher in the magnetic particle ELISA[30] (IC_{50} = 0.135 μg L^{-1}). This difference can be due to different BDE47 protein conjugates that were used in these formats. Another BDEs immunoassay[29] described a similar sensitivity as obtained in this study. Note that no limits are set yet for BDEs within the European Union (EU) and by U.S. EPA. Other immunoassays were developed for other indicators for PCBs, such as 2,3′,4,4′,5-pentachlorobiphenyl (PCB118),[31,41] but their sensitivities for dioxin-like PCBs were rather low.

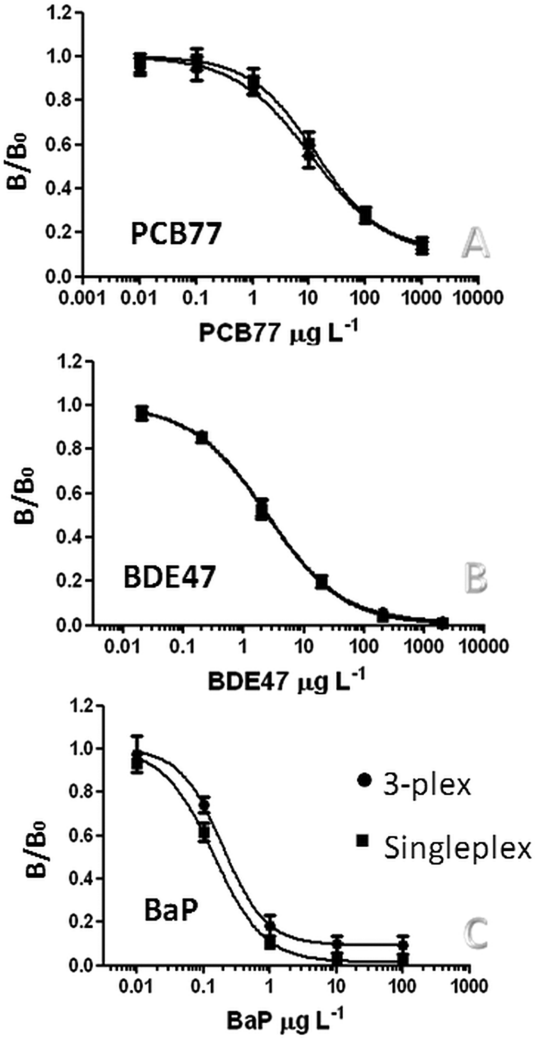

Figure 1. Dose−response curves obtained with the FCIA in three-plex (●) and single-plex (■) formats in buffer for the three main POPs representatives analyzed in this current study: (A) PCB77, (B) (BDE47), and (C) (BaP). The relative binding (B/B_0) was calculated by dividing the response (B) of each concentration by the maximum response obtained in a solution without the analyte (B_0). Solid lines show curves fitted with the four-parameters (4P) model. Each point represents the average of six replicates ± SD.

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Table 1. Three-Plex FCIA Characteristics in Buffer and Fish Extracts and Comparison to Previously Reported ELISAs for the Detection of the Three Target POPs in Buffer

target POPs	matrix	goodness of the 4P R^2 [a]	curve steepness (mL ng^{-1})[b]	IC_{50} ppb in the three-plex FCIA[c]	IC_{50} ppb in ELISAs
PCB77 (A)	buffer	0.9968	−0.8	20 ± 2	2−15[d]
	fish extract	0.9927	−0.6	55 ± 5	not measured
BDE47 (B)	buffer	0.9992	−0.7	2 ± 0.1	0.135[e]
	fish extract	0.9902	−0.7	2 ± 0.4	not measured
BaP (C)	buffer	0.9857	−1.3	0.4 ± 0.1	0.3[f]
	fish extract	0.9435	−1	4 ± 0.5	not measured

[a] Goodness of the four-parameter model fit to the calibration curve. [b] Calculated from the four-parameter fitted calibration curve. [c] The average half-maximal inhibitory concentration (IC_{50}) for each analyte extrapolated from six standard curves as the concentration of the analyte that provokes 50% inhibition of the maximum response. [d] Ref 34. [e] Ref 30. [f] Ref 32.

The specificities of the three single-plex FCIAs were determined by the assessment of the cross-reactivity (CR) pattern with the different target POPs selected on the basis of EU and EPA regulations and their structural similarities to PCB77, BDE47, and BaP. The percentage of CR was determined using the 50% displacement method,[42] and the data thus obtained are included in Table S-1 (in the Supporting Information).

Development of the Three-Plex FCIA. In the three-plex FCIA, the three single-plex assays were combined. However, this initially resulted in the PCB77 related cross-interaction of the PabPCB77 antiserum to the BDE47−BSA-coated microbeads. Using the purification procedure described in the Experimental Section, the binding of the purified PabPCB77 antiserum to the BDE47−BSA-coated microbeads could be strongly reduced from 3000 to 50 MFIs and was no longer PCB77-, BDE47-, or BaP-related.

The normalized PCB77, BaP, and BDE47 dose−response curves obtained in the three-plex and single-plex FCIAs are shown and compared in Figure 1. The low standard deviations (SDs; $n = 6$) indicate that the curves are highly reproducible. The sensitivities of the single-plex FCIAs and the three-plex FCIA for the analytes in buffer are similar. The curve fitting was done with four-parameter fitting, and R^2 varied from 0.98 to 0.99 (Table 1). The accuracy and precision of the dynamic ranges in the three-plex FCIA were determined by replicate analyses of PCB77, BDE47, and BaP curves. Known concentrations of each analyte (0−1000 $\mu g\ L^{-1}$) were assayed in different sets of wells in the same plate (intra-assay measurements; $n = 2$) and in different plates on different days (interassay measurements; $n = 6$). Results showed that the sensitivity of the individual assays in the three-plex FCIA format after the affinity purification of the PabPCB77 antiserum remained the same and is in compliance with the present established EU and U.S. limits.

Specificity of the Three-Plex FCIA. In addition to the sensitivity assessment of the newly developed three-plex FCIA for the marker POPs, CRs of the three different antibodies toward a panel of 51 POPs were determined and compared with previous results in Table S-1 in the Supporting Information. After the optimization process, no cross-interactions were displayed between the assays. The individual calibration curves were also tested in the final three-plex format, and the individual assays were specific for their own target analytes.

The CR patterns obtained with the three different assay formats (classic ELISAs and single-plex and three-plex FCIAs) were similar, with a few exceptions. The comparison between the single-plex FCIA and the ELISA[32] for the PAHs has been described previously.[36] No or low CR was obtained in all assays for the two- and three-ring containing aromatic compounds (Supporting Information Table S-1, parts A1 and A2). For the other tested food-related PAHs, the three-plex FCIA is less sensitive for BjF compared to the single-plex. These differences between the assays might be explained by the fact that the MabBaP used in the three-plex FCIA was an extra affinity-purified batch. The cross-reactivities of all assays for non-ortho and mono-ortho PCBs congeners were relatively low with only slight detection of one mono-ortho congener PCB105 and one non-ortho PCB126 in the three-plex FCIA (Supporting Information Table S-1, part B).

In the case of the BDEs (Supporting Information Table S-1, part C), a higher CR was shown for BDE99 in the ELISA than in the single-plex and three-plex FCIA; however, in FCIAs we used a different protein conjugate of BDE47. From our experience from previous work,[36] that can have a big influence on the sensitivity and specificity of the assay. 5′-MeO-BDE47 exhibits high sensitivity in all the assay formats. It seems that the methoxy derivative fits more closely to the hapten used to generate the antibody.[30] BDE28 exhibits slightly higher CR in both FCIAs. The low or no CR for the rest of the BDEs tested was comparable for all the immunoassays.

The developed three-plex FCIA in buffer has potential as a rapid screening assay, since it can detect several POPs simultaneously, such as the most abundant flame retardant BDE47 in fish, along with PCB77 belonging to the group of the most toxic dioxin-like PCBs and BaP, CHR, and many other PAHs belonging to the group of the eight European Food Safety Authority (EFSA) designated PAHs.

Applicability of the Three-Plex FCIA to Fish Samples and Aroclors. The direct measurement of POPs in high fat content foods is challenging for most analytical techniques, including immunoassays. POPs tend to accumulate in the fat tissues; therefore, simplified extraction and transportation into an immunoassay-compatible solvent (such as DMSO) is essential. To demonstrate the three-plex FCIA's applicability combined with a simplified sample preparation in a relevant food material, extracts of different fat content fish and different levels of PAHs, PCBs, and BDEs were analyzed (Table 2). The applied QuEChERS-like extraction technique relies on a favorable partition of POPs from the fatty sample material into the extraction materials (organic solvent and a mini silica column) and finally into DMSO. To study the influence of the silica minicolumn cleanup, we used aliquots of the same extracts before and after this cleanup in triplicate. The fish extracts after the cleanup step were also analyzed using capillary gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) and the levels of contamination

Table 2. Contaminated Fish Samples with Their Fat Contents, Levels of the Target POPs (BaP, PCB77, and BDE47) as Measured with GC/MS, and the Percentages of Inhibition of the Maximum Responses as Were Measured in the Three-Plex FCIA with (+) or without (−) the Cleanup

fishes	target POPs measured	fat content %	$\mu g\ kg^{-1}$ as measured in GC/MS	cleanup	% of inhibition of maximum response in three-plex FCIA
smoked trout	BaP	10	0.06	−	0
smoked trout	BaP	11	1	−	80 ± 2
smoked trout	BaP	14	5	−	80 ± 5
smoked trout	BaP	13	14.7	−	80 ± 3
trout	PCBs/BDEs	2	n.d.	+	0 ± 0.1
chub	BDE47	1.5	0.43	+	45 ± 2
chub	BDE47	2	4.93	+	56 ± 5
chub	BDE47	2	9	+	50 ± 4
chub	PCB77	1.5	1.95	−	22 ± 2

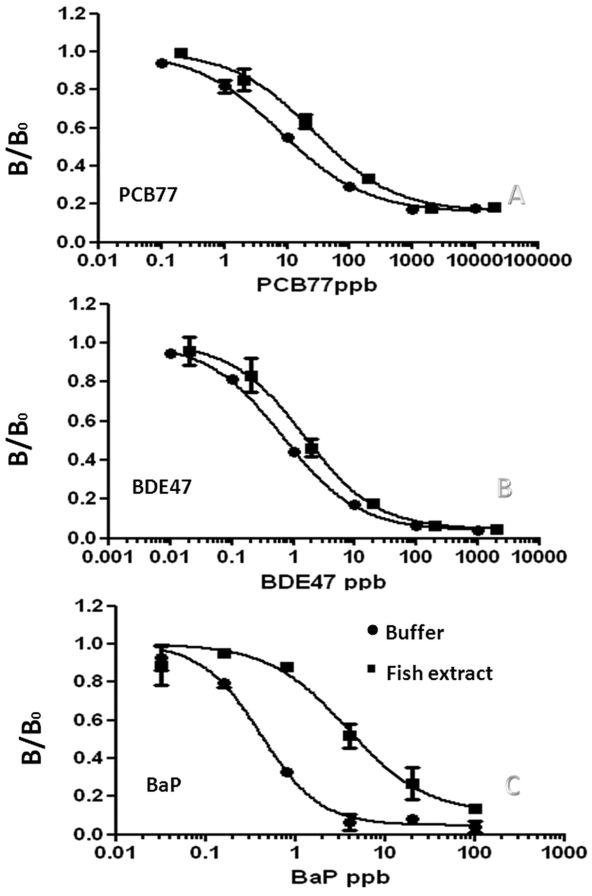

Figure 2. Dose−response curves obtained with the three-plex FCIA in buffer (●) and fish extract (■) for the three main POPs representatives analyzed in this current study: (A) PCB77 (fish extract (−) cleanup), (B) BDE47 (fish extract (+) cleanup), and (C) BaP (fish extract (−) cleanup). The relative binding (B/B_0) was calculated by dividing the MFI response (B) of each concentration by the MFI obtained in a solution without the analyte (B_0). Solid lines show curves fitted with the four-parameters (4P) model. Each point represents the average of six replicates ± SD.

for BaP, BDE47, and PCB77 as well as the fat contents are given in Table 2. However, in most fish extracts, more POPs were detected with the GC/TOF-MS than just the marker compounds, for example, BaA, CHR, CCP, BDE100, PCB153, etc.

Dose−response curves of BaP, PCB77, and BDE47 in buffer and blank fish extracts with no cleanup for the PCBs and PAHs and with silica cleanup for the BDEs were measured and compared in the three-plex FCIA (Figure 2). In general, the three-plex assay's sensitivity was not affected by the fish extract except for the BaP assay with a 10 times lower sensitivity and an IC_{50} of 4 $\mu g\ L^{-1}$ in matrix compared to the 0.4 $\mu g\ L^{-1}$ in buffer (Table 1). However, the PAH's FCIA is still adequate for screening smoked fish at the EU limit of 5 ng BaP per g wet weight of smoked fish and smoked fishery products; moreover, it can also detect more PAHs than just BaP. The maximum limit (ML) for the sum of dioxin and dioxin-like PCBs for fish and fishery products, excluding eel, is 8 pg of WHO PCDD/F-PCB-TEQ/g wet weight fish. The PCB77 toxic equivalency factor (TEF) value as stated for humans[43] is 0.0001, which results in a TEQ of 80 μg per kg fish. The IC_{50} value for PCB77 obtained in the three-plex FCIA was 55 ± 5 $\mu g\ kg^{-1}$ fish and thus well below the ML. For the emerging BDE contaminants, no limits are set by both EU and U.S. EPA. We obtained an IC_{50} of 2 $\mu g\ kg^{-1}$ of fish with a silica cleanup for BDE47.

The maximum responses (B_0) obtained in the three-plex FCIA for the blank fish extracts with or without cleanup were similar to the B_0 of the assays performed in buffer, with the exception of the PCB assay which showed an increase of 2500 MFIs without cleanup that was decreased after the cleanup. In the presence of PAH contaminants in the fish extracts, the decrease of the response was high (80% ± 3%), and even for the low-contaminated samples (Table 2), which indicates the presence of cross-reacting PAHs especially in the case of no extra cleanup. An average similar decrease was measured after the cleanup, but a high SD for the replicates of the three positive PAHs extracts indicates that the cleanup procedure was not yet very reproducible for PAHs (data not shown). Moderate decrease (45% ± 5%) of the B_0 was measured in the BDEs positive fish extracts after the cleanup (Table 2). For the positive fish extracts without cleanup, BDEs could not be detected in the samples. Most likely the residual fat in these extracts did not allow the BDEs to interact with the corresponding antibody. With the PCBs-contaminated fish we could measure a slight decrease of 22% ± 2% of the B_0, even without the cleanup step.

However, PCB77 is not the only dioxin-like congener present in food. PCBs were produced in the United States as standard mixtures known as Aroclors. Each Aroclor mixture has a unique dioxin and non-dioxin-like PCBs content,[44,45] and they are still abundant in the environment and subsequently in food. In this

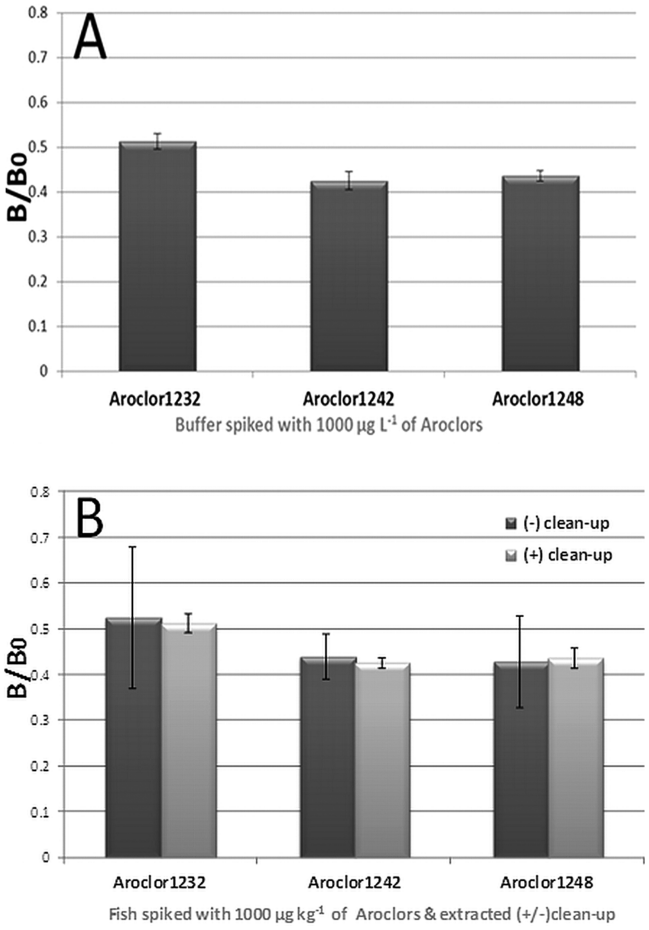

Figure 3. Relative inhibition (B/B_0) of the maximum MFIs (B_0) caused by the addition of 1000 ppb of Aroclor 1232, 1242, and 1248 to buffer ($\mu g\ L^{-1}$) (A) and fish ($\mu g\ kg^{-1}$) (B), after applying a concentration step of 2.5 (2.5 g of fish mL^{-1} of extract), as measured in the three-plex FCIA. The different Aroclors-spiked fish samples were extracted with (+) or without (−) cleanup using the simplified extraction procedure described in this paper.

study, we investigated the possible detection of the Aroclors 1232, 1242, and 1248 in buffer and spiked blank fish (Figure 3). Figure 3A shows 50−60% inhibition after the addition of 1000 $\mu g\ L^{-1}$ of the Aroclors to buffer. This inhibition corresponds to around 20−50 $\mu g\ L^{-1}$ of PCB77 equivalents (Figure 1). According to Van den Berg et al.,[43] the Aroclors contain 0.2−0.4% of PCB77, which corresponds to 2−4 $\mu g\ L^{-1}$ of PCB77 in the added Aroclors. This means that other congeners from the Aroclors cross-react with the antiserum. This can partially be explained by the contribution of the known cross-reacting congeners [PCB105, 126, 156, and 169 (Supporting Information Table S-1)]. Therefore, other structurally related congeners, such as PCB33 and PCB37 with chlorine positions attached to the 3 and 4 position of the benzene ring and present at much higher concentrations in the Aroclors,[45] may have contributed to this inhibition. The IC_{50} value for PCB77 in our assay is 20 $\mu g\ L^{-1}$, and the limit of detection (LOD) is around 2 $\mu g\ L^{-1}$ (Figure 1), so we can easily detect 100 $\mu g\ L^{-1}$ Aroclor in buffer. The total dioxin-like PCB fractions in the Aroclors 1232, 1242, and 1248 are 1%, 1.6%, and 4.9%, respectively, and the detectable concentrations of the dioxin-like fractions vary from 1 to 5 $\mu g\ L^{-1}$. With the three-plex FCIA, we could detect differences in responses between the extracts of the nonspiked blank fish and the spiked fishes with 1000 ppb of the three Aroclors with and without cleanup in which the concentration factor was 2.5 (2.5 g of fish per mL); however, without the cleanup the SDs of repeated analysis of the fish extracts were high. In the case of 100% recovery, the final concentrations of the Aroclors should be 2.5 times higher (2500 $\mu g\ L^{-1}$) compared to the buffer. However, for all fish extracts, the inhibited responses are similar (37−61%) to those in buffer, indicating losses during the extraction. The addition of 1000 $\mu g\ kg^{-1}$ of the Aroclors caused significant inhibition. This amount of Aroclor contains 10−50 $\mu g\ kg^{-1}$ dioxin-like PCBs, or 3−16 pg TEQ/g (with an average weighted TEF of 0.00032,[44]) which is around the maximum limit for dioxins and dioxin-like PCBs of 8 pg TEQ/g wet weight fish. Previously, another group[34] also tested the feasibility of an immunoassay to detect Aroclors in buffer but not in a sample material. The three-plex FCIA seems to be applicable for the simultaneous detection of several POPs in fish with various fat content and contamination levels.

CONCLUSIONS

This work describes the development and performance characteristics of a multiplex FCIA for the screening of POPs in buffer and fatty food material such as fish. For the first time, representatives of three main POP groups—BDEs, PCBs, and PAHs—can be detected simultaneously in fish by combining three different immunoassays in one format. Certainly, instrumental analysis offers the identification and quantification of individual POPs, but at high cost and time of analysis. The three-plex FCIA can rapidly screen for POPs contamination in food by analyzing about 40 samples in 2.5 h (including sample preparation) and, after further validation, can be a valuable prescreening tool for POPs in fish and other food and environmental samples prior to GC/MS. Furthermore, the developed three-plex FCIA meets the regulatory requirements of the EU and U.S. food safety authorities for PCBs and PAHs.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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